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TITLE: Elucidating the Mechanism of Gain of Toxic Function From Mutant C1 Inhibitor Proteins in Hereditary Angioedema

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14. ABSTRACT HAE is autosomal dominant. Cells, heterozygous for the SERPING1 mutation, express both mutant and WT C1INH proteins, however secreted C1INH levels are markedly lower than the expected 50%. This project sought to determine the mechanism responsible for the low C1INH levels. We developed novel techniques that will be useful in the study of HAE. We unequivocally demonstrated that mutant C1INH induces a dominant negative effect on wild-type C1INH. We also showed that mutant C1INH induce ER stress. Finally, we show that the GOTF is not restricted to natural HAE causing mutations but appears to be intrinsic to almost any disruption of the normal C1INH structure. Our findings suggest a novel impact of misfolded proteins on secretion of wild-type proteins. These findings also suggest that abrogating the GOTF should rescue normal protein secretion and ameliorate disease.					
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1. INTRODUCTION:

HAE is autosomal dominant. Cells, heterozygous for the SERPING1 mutation, express both mutant and WT C1INH proteins. HAE is clearly a loss-of-function disease. Plasma functional C1INH levels in symptomatic HAE patients, however, are very low - far less than the predicted 50%. We have shown that this is due, at least in part, to an additional acquired GOTF defect caused by the mutant protein that interferes with the secretion of WT C1INH. Our overall hypothesis is that mutant C1INH proteins exert a variable GOTF phenotype that inhibit secretion of WT C1INH protein and worsen disease severity. This hypothesis suggests that abrogating the GOTF should result in clinical benefit. Two aims will address complementary sub-hypotheses. Aim #1 will assess the mechanisms of the GOTF with a hypothesis that misfolding of mutant C1INH protein in the ER causes impairment of WT C1INH secretion. Elucidating the mechanism of the mutant C1INH-induced GOTF opens the possibility for new therapeutic approaches designed to abrogate the GOTF. Aim #2 will use a synthetic biology approach to assess the structural determinants of mutant C1INH-induced GOTF with a hypothesis that unique structural characteristics of C1INH make it more susceptible to GOTF than other serpins.

1. KEYWORDS:

Hereditary angioedema, C1 inhibitor, serpin, mutation, secretion, aggregates, misfolding

2. ACCOMPLISHMENTS:

What were the major goals of the project?

- 1) To define the mechanism how mutant C1INH proteins inhibit secretion of normal C1INH.
 - a) To identify the role of C1INH polymerization in the inhibition of secretion of normal C1INH (months 1-20; 50% completed)
 - b) To identify the role of ER stress in the inhibition of secretion of normal C1INH (months 12-32; 5% completed)
 - c) To identify whether abrogating the GOTF phenotype decreases evidence of C1INH polymerization or ER stress (months 24-36; 0% completed)
- 2) To analyze the structural requirements for the mutant C1INH protein-induced GOTF.
 - a) To assess the ability of alpha₁-AT mutations engineered into C1INH to induce a GOTF (months 1-24; 25% completed)
 - b) To assess the GOTF in chimeric C1INH/alpha₁-AT proteins with C1INH or alpha₁-AT mutations to induce a GOTF (months 6-36; 10% completed)

What was accomplished under these goals?

Aim #1

We demonstrated that we could detect C1INH polymers in transfected cells, and that mutant C1INH formed small polymers in transfected cells. We then concentrated on determining whether wild-type C1INH was trapped in these polymers. To accomplish this, we sought to detect the wild-type C1INH by labeling it with a FLAG-tag and using anti-FLAG antibodies to

specifically blot wild-type C1INH in the pathologic polymers.. A FLAG tag was placed into the wild-type C1INH cDNA located immediately 3' of the signal peptide. The FLAG-tagged C1INH protein was expressed and secreted and could be detected using anti-C1INH antibodies; however, we were unable to detect it using an anti-FLAG antibody. Suspecting that this problem was due to steric hindrance, we pursued multiple ways to overcome the problem, ultimately finding that insertion of a 3x-FLAG sequence between the 97th and 98th codon of the C1INH allowed detection of the labeled protein. The first 97 amino acids of C1INH define the N-terminal glycosylation domain and have been shown to be unimportant for C1INH function.

We then successfully demonstrated that transfection of COS-7 cells with either 3x-FLAG-WT-C1INH plus WT-C1INH or 3x-FLAG-WT-C1INH plus G162R-Mu-C1INH resulted in decreased secretion of the 3x-FLAG-WT-C1INH when cotransfected with the mutant cDNA. This was an important confirmation of our hypothesis and preliminary data, utilizing a more physiologic system. The results are illustrated in Figure 1 below.

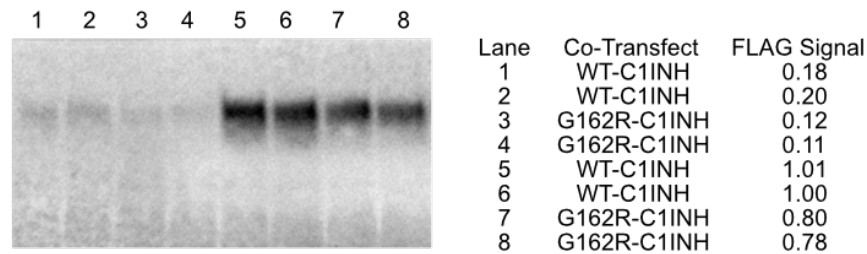


Figure 1. COS-7 cells were co-transfected with 3x-FLAG-WT-C1INH plus either WT-C1INH or G162R-C1INH. Secreted 3x-FLAG-WT-C1INH was visualized by immunoblotting with anti-FLAG. Lanes 1-4 = 24 hour supernatant; lanes 5-8 = 48 hour supernatant.

This assay provides a highly robust platform on which to test the impact of different mutant proteins on WT-C1INH secretion. We used it to look at the impact of various mutant C1INH constructs. The table below shows the results of this experiment.

Origin of mutation	Co-Transfection Construct	A1AT Equiv.	Replicate 1	Replicate 2
n/a	WT-C1INH	n/a	1.00	1.01
HAE	C183Y-Mu-C1INH	n/a	0.94	0.84
HAE	G162R-Mu-C1INH	n/a	0.57	0.57
HAE	V196D-Mu-C1INH	n/a	0.71	0.71

All 3 HAE disease causing mutations impaired the secretion of 3x-FLAG-WT-C1INH. We then examined whether there was evidence of the 3x-FLAG-WT-C1INH in the oligomers formed during co-transfection with G162R-Mu-C1INH by blotting with anti-FLAG. Cell lysates were separated on native blue gels. While the co-transfection with the mutant C1INH gave the expected increase in C1INH polymers, we did not see evidence of the 3x-FLAG-WT-C1INH in the higher molecular weight bands. This experiment was repeated numerous times, as we had

anticipated that the wild-type C1INH that we confirmed was not secreted would be found in the polymers.

Ultimately we accepted that this part of our hypothesis was incorrect. We then reasoned that the wild-type C1INH that was formed must be targeted for degradation in the proteasome in the presence of the mutant C1INH.

We therefore cotransfected cells with 3x-FLAG-WT-C1INH plus mutant C1INH cDNA in the presence or absence of a lactacystin, a proteasome inhibitor. As shown in figure 2, blocking degradation of C1INH by the proteasome resulted in a clear increase in the amount of FLAG-tagged C1INH detected in the cell lysate.

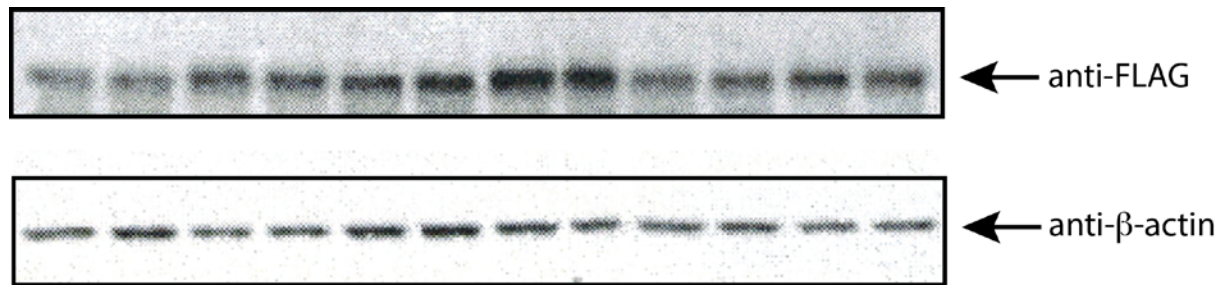


Figure 2. HEK293 cells were transfected with 3x-FLAG-C1INH plus either WT-C1INH (lanes 1-4), G162R mutant C1INH (lanes 5-8) or empty vector (lanes 9-12). Cells were treated with media alone (lanes 1-2, 5-6, 9-10) or lactacystin (lanes 3-4, 7-8, 11-12). Lysates were immunoblotted using anti-FLAG (top) or anti-β-actin (bottom). Lysates were immunoblotted using either anti-FLAG (top) or anti-β-actin (bottom).

Based on these results, we now conclude that mutant C1INH protein induces the GOTF with decreased secretion of wild-type C1INH by enhancing the proteosomal degradation of wild-type C1INH. We suspect that this is due to ER stress.

We have also looked for evidence of ER stress in cells transfected with mutant C1INH. We designed and optimized a real-time quantitative RT-PCR assay for BiP (Grp78). Treating COS-7 cells transfected with wild-type C1INH and then treated with brefeldin for 4 hours resulted in a 270% increase in BiP mRNA 48 hours after transfection and a 253% increase 72 hours after transfection. We observed, however, no change in the BiP levels in cells transfected with wild-type versus mutant C1INH cDNA. More surprisingly, we also did not observe any increase in BiP mRNA following transfection with α1-antitrypsin NHK, which has been previously reported to lead to increased expression of BiP.

We also assessed whether mutant C1INH would cause an increase in BiP protein. Figure 3 shows the BiP protein levels in cells transfected with either WT-C1INH or G162R-Mu-C1INH. The lysates were blotted for BiP, then stripped and re-probed for the housekeeping gene beta-actin.

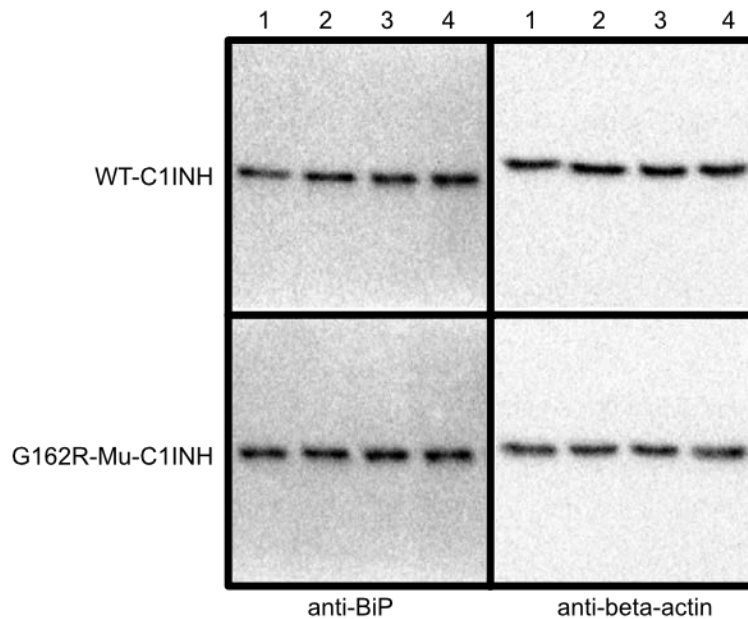


Figure 3. Cells were transfected with WT-C1INH or G162R-Mu-C1INH and the lysate recovered and blotted for BiP (left). Membranes were then stripped and reprobed for beta-actin (right). Lanes 1-2 used 1.5 ug of plasmid DNA while lanes 3-4 used 2 ug of plasmid DNA.

The cells transfected with the G162R-Mu-C1INH appear to have increased BiP (particularly when normalized to the beta-actin levels) compared to the cell transfected with WT-C1INH. The BiP level was 27% higher at 1.5 ug DNA and 14% higher at 2 ug. We also measured BiP mRNA by TaqMan real-time quantitative RT-PCR, and saw small increases in the mRNA level in cells transfected with G162R-Mu-C1INH.

In order to enhance the sensitivity of our measurements, we developed a promoter-reporter assay that uses the BiP promoter elements and a firefly luciferase reporter. Cells were also transfected with a Renilla luciferase reporter under the control of an endogenous promoter (PGL4.74) to normalize the results. Cells were cotransfected with wild-type C1INH plus the BiP-Firefly luciferase plasmid, the TK-Renilla luciferase plasmid, plus either wild-type C1INH, G162R C1INH. As a control we also cotransfected cells with wild-type A1AT plus either wild-type A1AT or mutant NHK A1AT (which is known to cause ER stress).

Co-transfection	Firefly luciferase	Renilla luciferase	Ratio	Fold Increase
Wild-type C1INH	182,447,728	70,714	2,580	
	201,994,160	70,589	2,862	
G162R C1INH	392,510,400	30,097	13,042	4.72
	470,603,200	37,152	12,666	
Wild-type α 1AT	113,137,152	35,348	3,201	
	98,634,424	35,799	2,755	
NHK α 1AT	196,457,680	30,761	6,387	2.24
	260,361,520	37,479	6,947	

As shown in the table, co-transfection with G162R mutant C1INH resulted in a 4.72 fold increase in BiP promoter activity compared to wild-type C1INH. In contrast, NHK A1AT, which is known to increase ER stress, resulted in a 2.24-fold increase in BiP promoter activity. Thus, we have evidence now that type I mutant C1INH proteins induce a GOTF by causing ER stress and degradation of wild-type C1INH in the proteasome.

We also asked whether type II HAE mutant proteins can also cause this gain-of-toxic-function. This question is of interest because the plasma C1INH levels are normal or near-normal in type II HAE. On the other hand, C1INH functional levels are as low in type II HAE as in type I HAE. If the type II mutants did not inhibit wild-type C1INH secretion, the potential relevance of the gain-of-toxic-function effect could be questioned. Figure 4 shows the results of transfecting COS-7 cells with FLAG-tagged wild-type C1INH cDNA plus either two different type I mutant C1INH cDNAs or two different type II mutant C1INH cDNAs. Forty-eight hours after transfection, the supernatant was collected and blotted for secreted wild-type C1INH using an anti-FLAG antibody. As can be seen, both type I and type II mutant C1INH cDNA caused a substantial decrease in the amount of secreted FLAG-tagged wild type C1INH. We have repeated this experiment several times with similar results. Therefore, we show that type II mutant C1INH protein can also result in a gain-of-toxic-function by decreasing the amount of wild-type C1INH that is secreted.

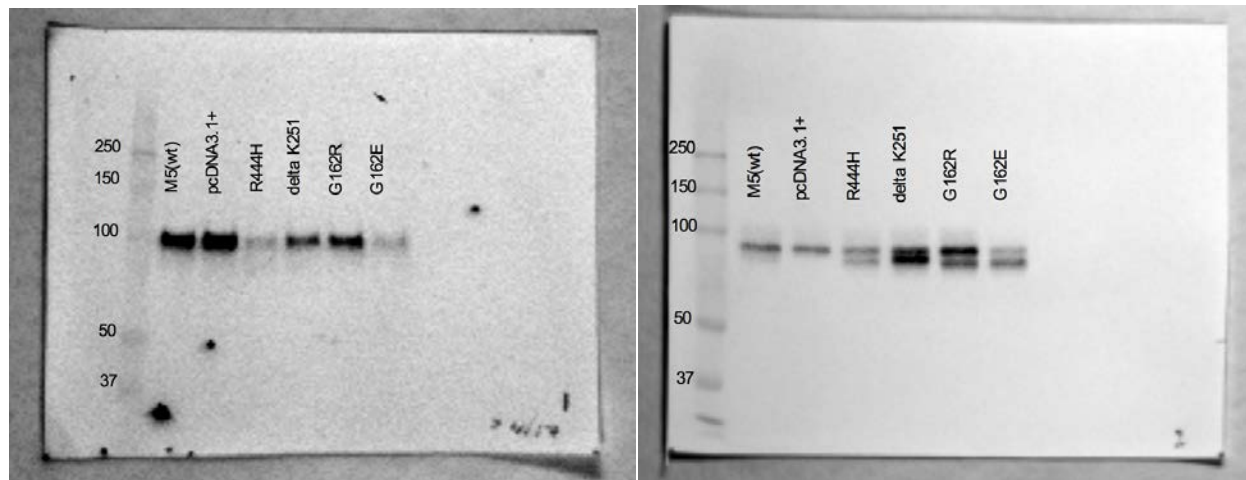


Figure 4. Transfection with FLAG-tagged wild-type C1INH plus untagged wild-type C1INH (lane 1); empty vector (lane 2); type II C1INH mutants (lanes 3-4) or type I C1INH mutants (lanes 5-6). Left: Secreted wild-type C1INH was detected using an anti-FLAG antibody; Right: Intracellular C1INH was detected using an anti-C1INH antibody.

We also examined the intracellular accumulation of C1INH protein in cells co-transfected with FLAG-tagged wild-type C1INH plus either the type I or type II HAE mutant C1INH. In this case, the blot was probed with anti-C1INH antibody. As shown in the gel below, transfection with FLAG-tagged C1INH plus WT-C1INH showed more intracellular C1INH than cells transfected with FLAG-tagged C1INH plus pcDNA empty vector. The total amount of

intracellular C1INH was increased in cells co-transfected with FLAG-tagged C1INH plus mutant C1INH, however the amount of intracellular C1INH was variable. We did observe that all of the mutant co-transfections displayed a prominent under-glycosylated or partially degraded form of C1INH that was minimally present in the absence of the mutant protein.

We plan to use the secretion of FLAG- tagged wild-type C1INH protein as the marker to detect effects of pharmacologic chaperones on the mutant C1INH GOTF. A barrier to interpreting the result is the variability in transfection efficiency from well to well. To overcome this problem, we assessed methods to standardize the transfection. The best results came from using a single transfection, and then aliquoting the transfected cells into individual wells, each of which could be treated with a separate dose or type of pharmacologic agent. The success of this approach is illustrated in the example below.

2.2 x10⁶ COS-7 cells were seeded in a 100mm cell culture dish, and grown till they were 80% confluent. Cells were then transfected with WT-C1INH using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. One day after transfection, the cells were detached and re-seeded into a 96-well culture plate. Forty-eight hours after re-seeding, the supernatant was harvested. C1INH protein in the culture supernatant was measured by ELISA. As shown in the table below, there was remarkable homogeneity in the secreted C1INH level.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.584	0.586	0.576	0.569	0.569	0.559	0.568	0.571	0.539	0.552	0.533	0.534
B	0.582	0.570	0.566	0.564	0.554	0.554	0.547	0.545	0.534	0.543	0.527	0.523
C	0.589	0.563	0.559	0.548	0.550	0.553	0.553	0.534	0.514	0.537	0.522	0.526
D	0.572	0.555	0.566	0.550	0.541	0.537	0.537	0.523	0.519	0.532	0.518	0.512
E	0.557	0.547	0.557	0.537	0.564	0.535	0.537	0.542	0.520	0.533	0.551	0.526
F	0.568	0.559	0.557	0.564	0.562	0.552	0.528	0.564	0.537	0.532	0.515	0.526
G	0.580	0.579	0.564	0.570	0.571	0.565	0.558	0.606	0.560	0.551	0.527	0.533
H	0.599	0.593	0.586	0.572	0.577	0.577	0.567	0.560	0.554	0.551	0.552	0.597

The mean \pm SD C1INH ELISA OD was 0.553 ± 0.021 , and the variance was 0.0004. This should allow us to sensitively detect any significant impact of pharmacologic chaperones on GOTF.

We also blocked function of the proteasome using a chemical inhibitor, lactacystin. Inhibition of the proteasome resulted in increased intracellular levels of wild-type C1INH protein (identified using anti-FLAG) in cells cotransfected with 3x-FLAG-wild-type C1INH plus mutant C1INH

Aim #2

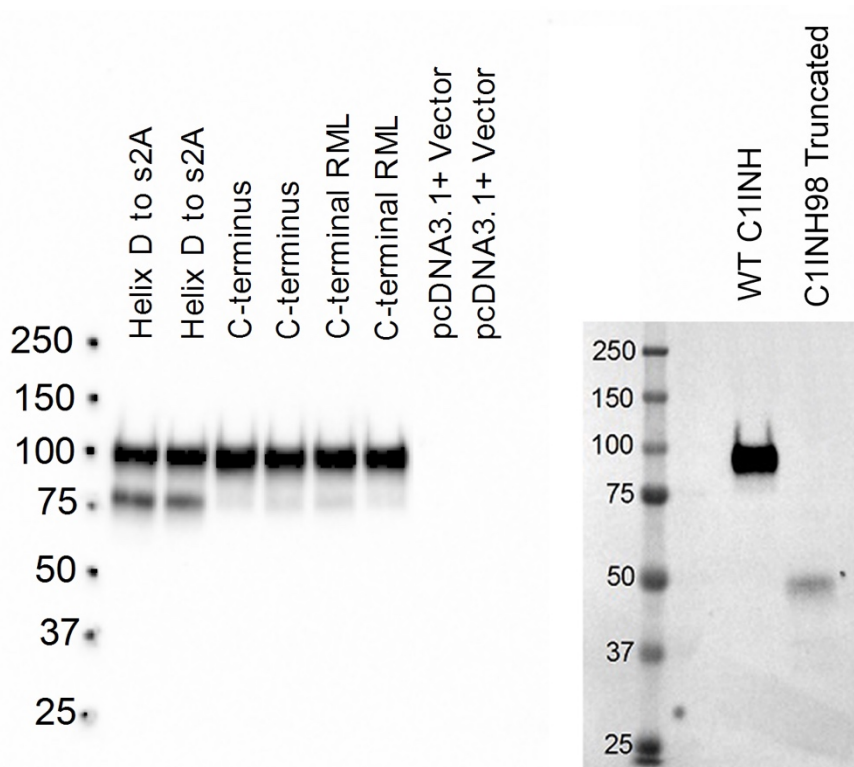
We successfully made novel C1INH mutants that mirrored the equivalent A1AT mutations that cause A1AT deficiency based on the homology between these two serpins to determine equivalent residues. Using these novel C1INH mutants, we asked whether we would still see a GOTF. We utilized the assay to measure secretion of 3x-FLAG-WT-C1INH. The results are shown in the table below:

Origin of	Co-Transfection	A1AT Equiv.	Replicate 1	Replicate 2
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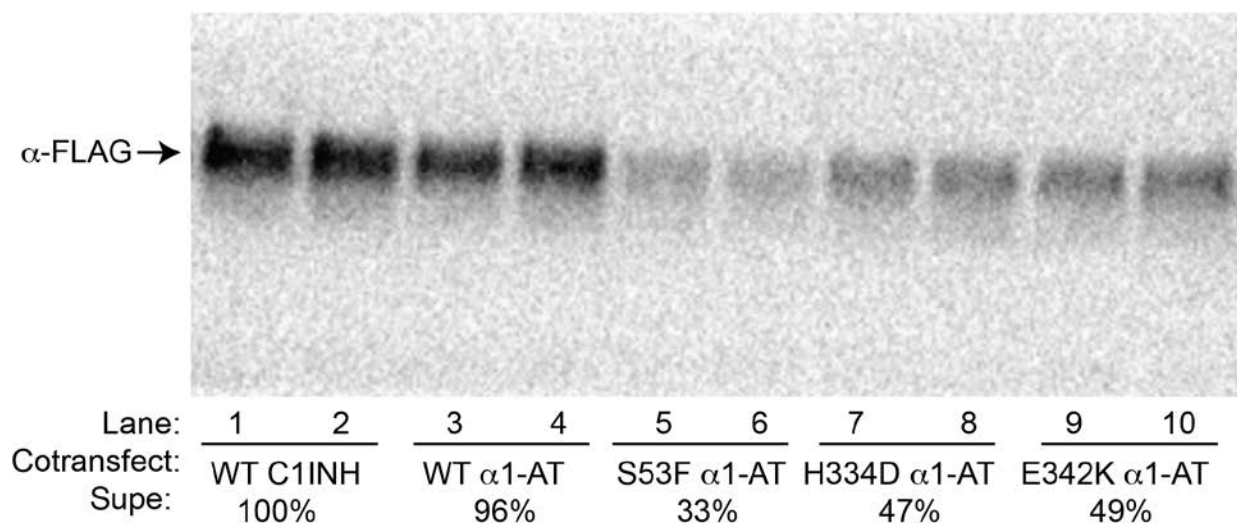
mutation	Construct			
n/a	WT-C1INH	n/a	1.00	1.01
A1AT	E429K-Mu-C1INH	Z	0.74	0.68
A1AT	H421D-Mu-C1INH	King's	0.66	0.67
A1AT	S148F-Mu-C1INH	Silyama	0.71	0.54

Surprisingly, we saw that introducing A1AT mutation equivalents into C1INH also hindered the secretion of 3x-FLAG-WT-C1INH. This is particularly notable because the A1AT mutations do not have an autosomal dominant effect on WT A1AT secretion. These results appear to support our hypothesis that the structure of C1INH is uniquely susceptible to GOTF, leading to the autosomal dominant pathophysiology.

We then constructed synthetic proteins in which domains were swapped out of α 1-AT and inserted into the C1INH protein. We have constructed and sequenced the N-terminal 96 amino acid deletion (C1 Δ .p.1-96). We have also engineered and sequenced: C1 Δ .p.82-198/ATins.p.88-108 in which the C1INH Helix D (aa182-198) is swapped out and replaced with the α 1-AT s2A sequence (aa88-108); C1 Δ .p.446-451/ATins.p.359-366 in which the unique part of the C1INH reactive mobile loop (aa446-451) is swapped out and replaced with the α 1-AT equivalent sequence (aa359-366); and C1 Δ .p.473-478/ATins.p.388-394 in which the C1INH C-terminus (aa473-478) is swapped out and replaced with the α 1-AT C-terminus (aa388-394). As shown below, these synthetic proteins are secreted. We are currently engineering 3x-FLAG tags into each of them.



We have also studied the impact of wild-type and mutant α 1-AT proteins on wild-type C1INH secretion. We asked whether this dominant negative effect would also be seen when cells were cotransfected with wild-type C1INH plus α 1-AT mutants. Notably, we saw no impact of the α 1-AT mutant proteins on 3x-FLAG-wild-type α 1-AT protein. Figure below shows that there was a substantial dominant negative effect seen when 3x-FLAG-wild-type C1INH was cotransfected with a variety of α 1-AT mutant proteins, but that no dominant negative effect was seen after cotransfection with α 1-AT wild-type protein.



Antitrypsin mutant proteins exert dominant negative effect on wild-type C1INH.

HEK293 cells were cotransfected with 3x-FLAG-wild-type C1INH plus either wild-type C1INH (lanes 1-2), wild-type α 1-AT (lanes 3-4), S53F mutant α 1-AT (lanes 5-6), H334D mutant α 1-AT (lanes 7-8), or E342K mutant α 1-AT (lanes 9-10). Secretion of wild-type C1INH was measured in the supernatant using anti-FLAG antibody and quantitated by densitometry.

We have also developed accurate techniques for measuring secretion of wild-type C1INH from type I HAE and normal subject monocytes. We see a marked decrease in secretion from the HAE type I cells, far less than 50% of normal. We are working to measure the secretion of wild-type C1INH from Type II HAE patients at the current time.

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

We are writing several manuscripts that we anticipate will be submitted soon.

What do you plan to do during the next reporting period to accomplish the goals?

We have requested a no-cost extension to allow us to finish these experiments.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Our studies have had a significant impact leading to a number of new observations that increase our understanding of HAE. First, we developed techniques that will be useful in the study of HAE. Second, we unequivocally demonstrated that mutant C1INH induces a dominant negative effect on wild-type C1INH. Third, we showed that mutant C1INH induce ER stress. And fourth, we show that the GOTF is not restricted to natural HAE causing mutations but appears to be intrinsic to almost any disruption of the normal C1INH structure.

What was the impact on other disciplines?

Surprisingly, we also found that α 1-AT mutants also inhibited secretion of wild-type C1INH. Based on this unexpected finding, we reasoned that C1INH plasma levels should be reduced in patients with α 1-AT deficiency. An old study in children with α 1-AT deficiency reported, in fact, that the plasma level of C1INH was significantly reduced in 75 PiZZ children compared to 35 control children (14%, $p < 0.01$). A similar reduction was found in 32 children with PiSZ (S mutation is E264V; 12%, $p < 0.01$). In the absence of a SERPING1 mutation and haploinsufficiency, this reduction in C1INH is not expected to have clinical significance, but it does support the concept that wild-type C1INH is highly sensitive to misfolded proteins and/or ER stress.

Similarly, the indirect impact is illustrated by Alzheimer's disease, a completely different protein misfolding disease. C1INH levels have recently been shown to be decreased in the plasma of Alzheimer's disease patients. While the basis for this reduction remains unknown, our preliminary data suggests that it could be due to susceptibility of wild-type C1INH to the misfolded amyloid protein. In the absence of haploinsufficiency, this doesn't cause angioedema; however, C1INH regulates factor XIIa and reduced C1INH levels may lead to enhanced activation of factor XII, which has been implicated in the progression of Alzheimer's disease.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

CHANGES/PROBLEMS:

Nothing to Report.

Changes in approach and reasons for change

There are no anticipated modifications of the plans relative to the SOW.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

No changes. The most recent IRB approval was in September 2017.

5. PRODUCTS:

- **Publications, conference papers, and presentations**

Zuraw, B.L., Herschbach J., Christiansen, S.C. Heterogeneity in the Secretion of Mutant C1 inhibitor Proteins. Manuscript being readied for submission.

Zuraw, B.L., Herschbach J., Christiansen, S.C. Type I Mutant C1 inhibitor Proteins are Retained in the Endoplasmic Reticulum. Manuscript being readied for submission.

Zuraw, B.L., Kansakar, S. Herschbach J., Christiansen, S.C. Mutant C1 Inhibitor Proteins Cause a Gain of Toxic Function with Decreased Secretion of Wild-Type C1 Inhibitor. Manuscript being readied for submission.

- **Inventions, patent applications, and/or licenses**

None.

- **Other Products**

None

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**What individuals have worked on the project?**

Name: Bruce Zuraw

Project Role: Principal Investigator
Research Identifier: bzuraw
Nearest person month worked: 3
Contribution to project: supervised project and other staff
Funding Support: None

Name: Marc Riedl
Project Role: Co-Investigator
Research Identifier: mriedl
Nearest person month worked: 1
Contribution to project: Building database of HAE patients who can be contacted for the study
Funding Support: None

Name: Jack Herschbach
Project Role: Research Technician
Research Identifier:
Nearest person month worked: 12
Contribution to project: constructed all plasmids and performed mutagenesis
Funding Support: None

Name: Astrid Doerner
Project Role: Project Scientist
Research Identifier:
Nearest person month worked: 12
Contribution to project: Stress responses and monocyte studies
Funding Support: None

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No

What other organizations were involved as partners?

Nothing to Report.

7. SPECIAL REPORTING REQUIREMENTS: None

8. APPENDICES:

None